

Standardization of Microchemical Methods and Apparatus

STANDARDIZATION is not new in macrochemistry, but it is new in microchemistry. Microchemistry is a mere child by comparison with macrochemistry, being only 40 years old. The growth and popularity that microchemistry has enjoyed in this country are justified, for it has met a great need in analytical chemistry. At first the procedures and apparatus adhered closely to those of the leaders in this new field of chemistry, but some microchemists have had no contact with the pioneers, and modi-

fications have crept into both the microprocedures and the apparatus. This is a healthy trend, but it also presents a problem, because many of the new or modified procedures or apparatus give the desired results only in the hands of those who proposed them. Why, no one knows, except that the specifications of some empirical condition have been omitted. There is nothing new in such an occurrence; it had been experienced in nearly every branch of chemical analyses. In macroanalysis, this problem has been re-

The paper presents a review of the work of the two AMERICAN CHEMICAL SOCIETY committees for the standardization of microchemical apparatus, and of the Association of Official Agricultural Chemists referee work on standardization of microchemical methods. An account of the AOAC referees' studies on methods for determination of nitrogen, carbon, and hydrogen is given, together with a statistical analysis of their data. It was demonstrated that standard microchemical methods can be developed by collaborative study from methods already in use, and that such standard procedures are usually free of conditions that must be arbitrarily and meticulously followed.

ceiving attention for many years, by such groups as the Association of Official Agricultural Chemists, the American Society for Testing Materials, the Pharmacopeia, the American Oil Chemists' Society, and the American Petroleum Institute.

There was an early realization that it would be necessary to standardize on certain pieces of microapparatus, and this resulted in the formation of a committee by the AMERICAN CHEMICAL SOCIETY in 1937, with G. L. Royer as chairman, to recommend standard microapparatus. Results of this committee's work, published in 1941 (5) and 1943 (3, 4), made available the best in design of many pieces of microequipment, and there was reasonable assurance that such apparatus when purchased would conform to the recommended specifications. After this committee terminated its work, improvements were made, and before long it was realized that standardization of microchemical apparatus should be brought up to date. Consequently a new ACS committee, with Al Steyermark as chairman, was set up by the Division of Analytical Chemistry in 1947. This committee is still active. Its first work (6) was to bring up to date the recommendations of the previous committee and then to recommend specifications for new items (7).

About the time the first committee on apparatus was writing its specifications, another small group began studies leading to the standardization of microchemical methods. E. P. Clark was largely responsible for this work, which was sponsored by the Association of Official Agricultural Chemists. His work was getting well under way when it was terminated by his death.

In 1948 the standardization of microchemical methods was again undertaken. Referees were appointed by the Association of Official Agricultural Chemists, with the author as general referee. By this time, some 40 microchemical methods were in common use, with all manner of modifications, yet no one had any assurance as to which method or modification could be relied upon. The new referees wanted all American microchemists to participate in the work leading to the standardization of microchemical methods, and canvassed them to determine the order in which they wished the different methods to be selected for study. Of the 36 methods voted upon by microchemists representing 73 laboratories, the carbon and hydrogen method received the greatest vote, 114 (on a weighted-point count system), followed by Dumas nitrogen, 69; Kjeldahl nitrogen, 56; sulfur, 39; and chlorine, 33. This was particularly revealing; the determination of carbon and hydrogen is the oldest and the best known microprocedure, and yet microchemists were sufficiently dissatisfied with the method to request that it be improved or standardized.

COLLABORATIVE WORK

The current collaborative work therefore began with the study of three methods—for carbon, hydrogen, and nitrogen. Both the Kjeldahl and the Dumas procedures for nitrogen were studied. The samples, nicotinic acid and benzylisothiurea hydrochloride, were selected because they are stable and nonhygroscopic, and because they differ considerably in constitution and ease of decomposition. Because there were no standard procedures for carbon and hydrogen, the collaborators were asked to determine them by their own procedures. The nitrogen analyses were to be done by their own micro-Kjeldahl and Dumas procedures and also by the

tentative AOAC micro-Kjeldahl method. It was hoped that a statistical analysis of the analytical results and the description of the method used by each analyst would identify the important variations of each method and apparatus.

In a collaborative study of this type, it was obviously impossible to follow the old "classical" design of experiments, in which all variables but one are held constant and the effect of the one variable is determined. Recently the classical design has been shown by Wood (11) to be inferior to the "factorial" design, in which several factors are varied systematically, so that the total effect of the different variables is observed. In this way the effect of varying *A* when *B* and *C* are at different levels or varying *B* when *A* and *C* are at different levels can be determined.

In the first year's work (9), the results for the Kjeldahl analyses were the most striking. When plotted, the analytical values for *s*-benzylthiourea hydrochloride produced a histogram showing a fairly normal distribution of the values around the mode which falls upon the theoretical value, 13.82%. The mean, 13.79, is only 0.03% less than theory, and the standard deviation is 0.16. This small standard deviation and high accuracy were expected, because in this compound the nitrogen exists in the amino form, which is easily converted to ammonia.

The values of nitrogen obtained for nicotinic acid, a compound with a heterocyclic ring nitrogen structure, produced a histogram that was not normal and was skewed toward the low values. Seventy-five values ranged from 0.5 to 11.5% as compared with a theoretical value of 11.38. These data did not lend themselves to statistical treatment, but they demonstrated a sharp division of values, half being near the theoretical value. A histogram in which were included only the data of those collaborators whose values were all above 11% gave a plot of more nearly normal distribution and, although the mode fell on the theoretical value, the mean was considerably lower. This indicated that some of the collaborators were using a method that gave very accurate results for C—N—C bonded nitrogen in a ring structure. The referees had meanwhile observed (1, 8) that the time and temperature of the Kjeldahl digestion were critical and low temperatures or a short digestion time contributed to low values, thus accounting for the skewness of the original plot. Consequently, in the 1949 collaborative studies (10), the time and temperature of digestions were specified to conform with these observations. When plotted, the results of the nitrogen analysis of nicotinic acid in the 1949 collaborative studies showed a mode only 0.03 below theory, and the standard deviation for 83% of the values was only 0.103%. Similar results were obtained for tryptophan, where the mean was only 0.06% below theory, with a standard deviation of 0.29%. The method proved acceptable as the standard for amino and C—N—C bonded nitrogen.

DUMAS NITROGEN

The same group of collaborators participated in the analysis of nicotinic acid by the Dumas procedure, and again their results when plotted did not represent a normal population. The mean, 11.20%, was only 0.18 below the theoretical value of 11.38%, however, and the standard deviation was only 0.235%. The cause of the skewness—that is, the large number of classes occur-

ring below theory—was therefore investigated. Information supplied by the collaborators revealed that most of the low values were obtained when the long furnace was heated at temperatures below 650° C. Consequently the nitrogen values for nicotinic acid obtained by methods using temperatures above 650° C. were tested, and they were found to be representative of a normal population.

CARBON AND HYDROGEN ANALYSES

Although the principles involved in the determination of carbon and hydrogen are the same in all procedures, the details may have as many variations as there are chemists performing the analyses. The referees consequently did not feel that they could establish a recommended procedure; instead, as in the nitrogen analyses, each analyst was asked to perform the analysis according to his own procedure. In this study (9) 25 analysts, representing 19 laboratories, reported 111 carbon and hydrogen values. These results showed that a majority of the carbon values were higher than the theoretical value, 58.53, and that the mean value was 58.66%, with a standard deviation (S , the variation about a mean) of only 0.244%. This indicated that 67% of these carbon values fell within $\pm 0.244\%$ of the mean. The important fact demonstrated by these carbon values was that although the mean was 0.13% higher than the theoretical value, the mode fell on the theoretical value, indicating that there was a good possibility of establishing a method that would give high accuracy as well as good precision.

In studying the carbon procedure, the following facts were recognized: All methods were generally the same, and although no two methods were identical, each step in any one method was in general similar to the corresponding step in several other methods. Therefore, the carbon values for each sample were divided into two groups, one group representing the results obtained by a certain operation for one step in the determination, and the other group representing values obtained by a different operation for the same step. These two groups of data were treated statistically to determine whether or not there was a significant difference in the results obtained by the two operations. This comparison was made for the following: semimicro (10 to 30 mg.) vs. micro (2 to 10 mg.) sample weights; electrical vs. gas sample burner; mechanical vs. hand-operated sample burner; air-conditioned vs. non-air-conditioned laboratories; balance in air-conditioned balance room vs. balance adjacent to the furnace. In nicotinic acid analyses, the only significant difference in the resulting values for carbon was in the use of semimicro vs. micro samples. The means of the values for these two procedures were 58.52 and 58.69, whereas the standard deviations were 0.198 and 0.239, respectively.

Other comparisons included: oxygen supply (pressure plus aspiration vs. pressure only); choking plug vs. no choking plug; replacing oxygen in absorption tubes by air vs. not replacing it; wiping absorption tubes vs. not wiping them; quartz for combustion tube vs. borosilicate glass; and catalyst copper oxide plus platinum vs. all others.

In considering the effect of air conditioning, the data had to be evaluated with the greatest reservation because the degree of control of the temperature and humidity and the means of air distribution were not known. Furthermore, neither actual conditions of the non-air-conditioned laboratories nor seasonal fluctuations were known, and analysts' statements indicated that these data could not be treated as though they represented a true subgroup. This was best demonstrated by the collaborating analysts' comment: "Our laboratory is not air-conditioned and we do not ordinarily attempt to run carbon and hydrogen in the summer for this reason," or "... I delayed starting the work until July hoping that we would finally get some warm, dry weather which would give fairly constant temperatures and not too high humidity."

A simple test that indicates when a marked difference in ac-

curacy in results obtained by the pairs of procedures occurs was the application of a new graphic test suggested by John Tukey (2) of Princeton University. This test indicated that in general the values of the microprocedure were higher than those obtained by the semimicroprocedure. The Student's t test was applied to these data to determine whether this apparent difference was significant. Student's t calculated from these data was 2.24; 2.03 is critical t for the 5% level. Therefore, the difference at the 95% level was significant. As a significant difference in the accuracy of results obtained by these two methods had been established, this variable was eliminated from subsequent comparison to avoid bias. Consequently the results of the microprocedure were treated separately.

By using only microprocedure values, the effect of wiping or not wiping the absorption tubes before weighing was analyzed by the Tukey graphic procedure. This test showed that wiping had a tendency to yield high results, and from the t test this difference was found to be significant at the 90% level. Similar treatment of the data indicated that replacement of the oxygen in the absorption tubes also caused high results. All other variations caused no significant difference in the accuracy of results. All the data were re-examined to see whether after an adjustment for the effect of each of these variables there still existed a significant difference in the results.

The effect of these variables on the precision for all the results was also determined. This method, also based upon a suggestion by Tukey, consisted of determining the variance of each analyst's carbon values, taking the logarithm of these, and again applying Student's t test as if they were means. These statistical investigations indicated that none of the variables exerted any appreciable influence on the precision of the method. Therefore, only those variables of each pair of conditions should be retained which influenced the accuracy or contributed to simpler operation or manipulations. These studies indicated that the procedure for carbon and hydrogen should include electric furnaces with mechanical operation, quartz or Vycor combustion tubes with a filling of only copper oxide plus platinum catalysts and silver wire or ribbon, no choking plug, pressure only for oxygen flow, no treatment of the absorption tubes other than to allow them to equilibrate before weighing, and use of 10- to 15-mg samples.

These studies showed that by collaborative study and statistical analysis it is possible to develop a standardized procedure wholly or in part from methods already in use. The standard method should not be set up arbitrarily and then approved by collaborative test. Instead, it should be developed by statistical analysis of data obtained by collaborative study. Conditions of the standard method should be so well defined and so adjusted that it is unnecessary to specify arbitrary conditions that must be meticulously followed. With the best in microchemical apparatus design readily available, together with standardized microchemical procedures, microchemistry should find ever-increasing use.

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